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Mutated in Sporadic Breast Cancer

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| 13. ABSTRACT (Maximum 200) <p>This report serves as the annual report for the period beginning July 1, 1996 for the project entitled "A Mouse Model for the Cloning of a Tumor Suppressor Gene Mutated in Sporadic Breast Cancer. This report states the experimental progress made and proposes a modification of an original specific aim. This modification serves to facilitate the speed of the research and substantially reduces the numbers of mice to be used in the study. Significant progress has been accomplished toward the goal of the project. Specifically, 2 replacement and 4 insertion vectors have been constructed and electroporated into Embryonic Stem cells. Homologous recombination events of these vectors were identified by Southern analysis. Cre mediated recombination events resulting in deletions of a portion of chromosome 6 were identified and confirmed by Southern analysis. Embryonic Stem cell lines containing deletions of a portion of chromosome 6 were microinjected into blastocysts and chimeric mice have been obtained. These essential preliminary experiments will allow for the production of mice and primary cell lines heterozygous for the deletions within chromosome 6. These reagents will allow for the identification of the tumor suppressor gene that resides within the deletion interval through an <i>in vitro</i> retroviral immortalization assay.</p> | | | | | | |
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FOREWORD

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Introduction

Cancer, stated most simply, results from the abnormal proliferation of cells. Cancer is a polygenic disease frequently requiring a number of independent mutations. A fundamental goal in studying any genetic disease is the identification of the causal gene or genes.

Alterations in dominant oncogenes and tumor suppressor genes are believed to be critical in the multistep process leading to the development of tumors¹⁻³. Gain of function mutations or aberrant expression of genes can lead to the stimulation of abnormal cellular proliferation in a dominant fashion. These genes are known as dominant oncogenes and have been shown to function at a variety of positions in signal transduction cascades. A large number of genes of this class of oncogenes have been cloned. A second class of oncogenes has been identified and is characterized by the loss of gene product function resulting in the loss of cellular growth control^{4,5}. These genes have been termed tumor suppressor genes and are recessive oncogenes; by definition requiring mutations in both alleles for neoplastic progression. Examples of this class of oncogene include *p53*, *RB*, and *APC*.

Many dominant oncogenes have been cloned through gain of function assays⁶. However, identification of tumor suppressor genes remains difficult due to the recessive nature of the mutations in these genes. Nonetheless, a small number of tumor suppressor genes have been cloned⁷⁻¹⁶. Some tumor suppressor genes are associated with autosomal dominant human familial cancer susceptibility syndromes that facilitate positional cloning strategies. The autosomal dominant genetics observed in these syndromes is misleading as the clinical phenotype results from a somatic mutation in the second allele of the

gene. This somatic mutation occurs within the context of an inherited loss of function mutation within the first allele. The mutations within both alleles result in the abrogation of the gene product function resulting in the clonal expansion of a single cell into a tumor. Genetic analysis of these syndromes is facilitated by a high degree of penetrance and the tissue specificity of the primary neoplasia. However, when penetrance is low or the tissue specificity is poor identification of an inherited component is complicated by the high incidence of sporadic disease. Thus, the characterization of a large number of tumor suppressor genes are refractory to standard genetic analysis in pedigrees.

Breast cancer is a very common malignancy with an estimated incidence of greater than 10% of all women during their lifetime. Furthermore, breast cancer patients accounted for 19% (1.145 million) of the total number of patients worldwide (7.6 million) affected by cancer in 1985^{17,18}. While the vast majority of breast cancers appear to be of sporadic origin, inherited mutations in several genes are associated with increased risks of developing breast cancer in women. This genetic predisposition may be responsible for 10% of breast cancers. Two such susceptibility genes have recently been identified, the *BRCA1* gene¹⁹ and the *BRCA2* gene²⁰⁻²².

Germ line mutations in the tumor suppressor gene *p53* cause the cancer predisposition syndrome known as Li-Fraumeni²³. While Li-Fraumeni individuals display many types of cancer affected women frequently develop breast tumors demonstrating the importance of *p53* function in this disease. Heterozygotes for mutations in a fourth gene, ataxia telangiectasia (AT) exhibit an increased risk of breast cancer in women²⁴. While the elucidation of the function of these breast cancer

familial predisposition syndrome genes will undoubtedly reveal insights into breast neoplasia our understanding of tumor suppressor gene involvement in the etiology of sporadic breast cancer remains poor.

Cytogenetic techniques have been utilized to identify possible locations of recessive tumor suppressor genes. The observation of a high frequency of deletions or inversions of part of a chromosome are indicative of the inactivation of a nearby tumor suppressor gene during neoplastic progression²⁵. The analysis of polymorphic genetic markers to identify loss of heterozygosity (LOH) can be used to screen for genetic alterations of tumor DNA and can identify very small chromosomal alterations²⁶. Analysis of known genes within a minimal region defined by the LOH analysis may be informative as to the putative identification of the tumor suppressor gene. However, absent such serendipity, the identification of the authentic tumor suppressor gene is problematic.

LOH analysis of DNA extracted from breast tumors using polymorphic genetic markers has identified a number of frequent alterations in human chromosomes; chromosomes 1p, 1q, 3p, 7q, 13q, 17p, 17q, and 18q²⁷. The majority of the LOH regions identified remain poorly resolved. However, detailed analysis of some of these regions have mapped several putative tumor suppressor loci to relatively small regions of the chromosomes. For example, using this type of analysis a 120kb interval between *D17S846* and *D17S746* distal to the *BRCA1* locus was indicated to contain a tumor suppressor gene²⁸. An additional LOH locus has been identified within a 2cM interval on chromosome 7q31.1-7q31.2²⁷. LOH analysis of DNA extracted from breast tumors revealed the loss of heterozygosity in 26 of 31 (83%) cases studied within the 7q31.1-7q31.2 interval, a very high frequency²⁹. Deletions of

chromosome 7 are common with many different types of tumors including; ovarian cancer, gastric carcinomas and malignant myeloid disorders³⁰⁻³⁶. Additionally, introduction of human chromosome 7 into a murine-derived squamous cell carcinoma cell line delayed the onset of tumors by 2-fold to 3-fold and in some cases can even suppress completely the tumorigenic potential of the squamous cell carcinoma cell line³⁷. Moreover, the insertion of human chromosome 7 into an immortalized human fibroblast cell line having LOH in the segment 7q31.1-7q31.2 resulted in the restoration of senescent properties to the cells³⁸. These data are consistent with the existence of at least one tumor suppressor gene on chromosome 7q31.1-7q31.2.

Mice can function as an in vivo assay for tumor suppressor gene mutation.

Our laboratory was the first to describe in mice that the effect of loss of function mutations in the tumor suppressor genes *p53*, *Rb* and *Xpc* result in a tumor susceptibility phenotype³⁹⁻⁴¹. Mutations in six known tumor suppressor genes have been reported in mouse models³⁹⁻⁴⁷. Mutations in *p53*, *Rb*, *mApc*, *Xpc* and *Nf1* are associated with a predisposition to tumor development^{39-45,47}. This data confirms the tumor suppressor function of these genes in mice. *p53*, *mApc*, *Xpc* and *Nf1* heterozygous mice display a very similar spectrum of tumors in comparison to patients with these mutant genes. However, mutations in *Rb* display different types of tumors in mice and humans. Furthermore, knockout mice homozygous for a deficiency in the α -inhibin gene product develop tumors in the gonads, revealing an unsuspected tumor suppressor function for this gene^{48,49}. These data indicate that the

mouse can serve as an *in vivo* indicator of tumor suppressor gene function and frequently display similar tumor types as the human predisposition syndromes.

Experimental Methods, Assumptions and Procedures

Hypothesis/Purpose

The objective of this proposal is to clone the mouse homologue of the tumor suppressor gene(s) involved in breast neoplasia identified by LOH analysis of human breast tumor DNA to reside on chromosome 7 within the interval 7q31.1-7q31.2. This proposal exploits the human-mouse syntenic relationship of human chromosome 7q and mouse chromosome 6 (See Figure 1) and utilizes the tumor susceptibility of mice that are heterozygous for tumor suppressor genes.

Primary fibroblast and mammary epithelial cells will be generated from mice bearing the deletion within chromosome 6. These cells will be infected with a retrovirus to inactivate the remaining tumor suppressor gene. The inactivation of this gene is likely to lead to the immortalization of the infected cell which will allow for the identification of the tumor suppressor gene. The tumor suppressor gene thus tagged by the retroviral insertion will then be cloned by established techniques. The identification of this tumor suppressor gene will lead to characterization of the function of this gene. These characterizations may reveal fundamental insights into the mechanism of a large fraction of sporadic breast cancers. Ultimately these studies may lead to

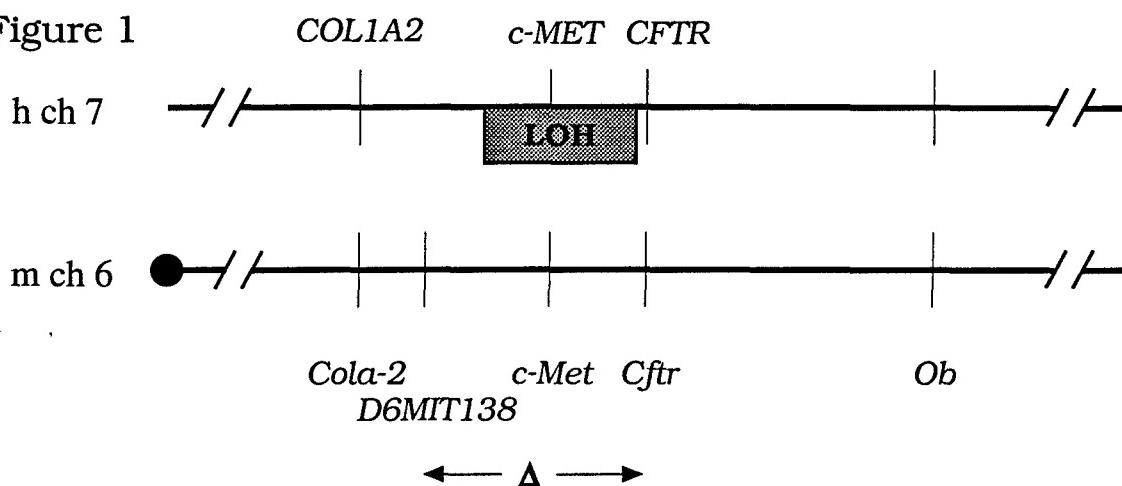
important diagnostic assays and novel chemotherapeutic or gene replacement treatments.

Technical Objectives

Objective 1. Construction of mice with tumor suppressor gene deletions.

Recently a tumor suppressor gene, involved in the development of breast cancer, has been linked to a 2cM region on human chromosome 7 within the interval 7q31.1-7q31.2²⁷. A deletion has been constructed so as to delete the portion of mouse chromosome 6 that is syntenic to human 7q31.1-7q31.2 (See Figure 1). This deletion has been established in a mouse Embryonic Stem (ES) cell line. The resultant ES cells are being utilized to generate mice heterozygous for the deletion.

Figure 1



Objective 2. Analysis of the deletion allele of mouse chromosome 6: propensity for tumor formation.

The loss of one allele of a tumor suppressor gene frequently leads to a dominant susceptibility or predisposition to tumorigenesis. The

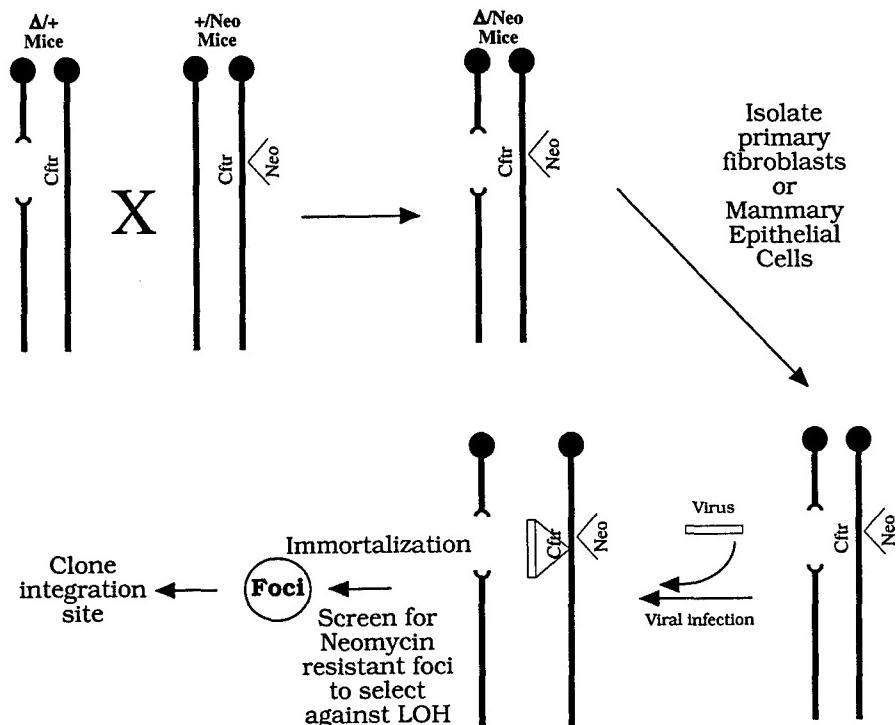
deletion of the tumor suppressor gene contained within the interval deleted on chromosome 6 is expected to result in an increased rate of tumor formation. Mice heterozygous for the deletion on chromosome 6 will be carefully observed and compared to a control wild-type mouse population for rate of tumor formation and tumor type. Additionally, mice heterozygous for the deletion on chromosome 6 will be bred with mice carrying a deficiency in the tumor suppressor gene *p53*. Offspring heterozygous for the deletion on chromosome 6 and the deficiency in *p53* will be bred with *p53* deficient mice to yield progeny heterozygous for the deletion on chromosome 6 and either homozygous or heterozygous for the *p53* deficiency. These mice will be observed for tumor formation relative to controls with an expectation of an increased susceptibility.

Objective 3. Retroviral mutagenesis, tagging and cloning of the tumor suppressor gene.

This proposal originally proposed to use the mouse mammary tumor virus (MMTV) to infect mice heterozygous for the deletion within chromosome 6. MMTV is a retrovirus, passed from mother to pup, that can integrate into the sole remaining allele of the tumor suppressor gene. This integration event will result in a deficiency of the tumor suppressor gene product function and is expected to lead to the clonal expansion of the mutated cell into a tumor. Integration events that disrupt the tumor suppressor gene were proposed to be identified using standard molecular biology techniques. However, to date, in control animals we have found the rate of tumor formation using the *in vivo* MMTV retroviral model system to be very low. Therefore, retroviral mutagenesis will be performed *in vitro* to circumvent the low frequency of tumor formation in

the *in vivo* model and in order to minimize the number of animals needed for the completion of this study (See Figure 2).

Figure 2



Primary fibroblast and primary mammary epithelial cells, when cultured *in vitro*, undergo a finite number of cell divisions. However, a number of genetic alterations can lead to the "immortalization" or indefinite growth of these cells. Such alterations include the loss of function of tumor suppresser genes, such as p53, and the transformation of proto-oncogenes. We will utilize this potential to identify the tumor suppressor gene within the deletion interval of chromosome 6.

Primary fibroblast and primary mammary epithelial cells will be cultured from embryos that are heterozygous for the deletion of

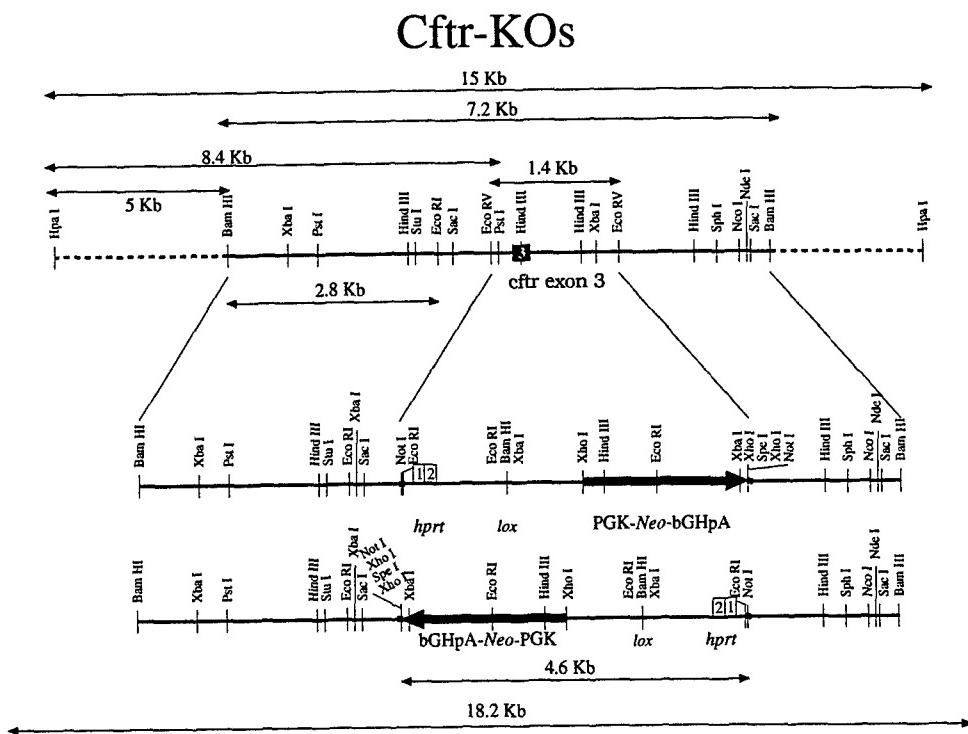
chromosome 6 and that carry a neomycin resistance gene at the *Cftr* locus of the other chromosome 6. These primary cells will be infected with one of two retroviruses that are designed to express resistance to puromycin when the virus has integrated into an expressed gene and to inactivate the gene into which the virus has integrated. Infected cells will be cultured with media containing both neomycin and puromycin and immortalization foci identified. The continued selection for the presence of the neomycin resistance gene at the *Cftr* locus will be used to select against LOH at this region; a problem this laboratory has experienced and which masks informative events. Retroviral integration sites will be cloned from the immortalized cell lines. Genomic sequences will be used to probe YACs that cover the deleted region of chromosome 6. Genomic sequences identified as originating within the deletion interval of chromosome 6 will be sequenced and analyzed.

Construction of mice with deletions for the tumor suppressor gene.

Targeting the first deletion end point. The *Cftr* gene was chosen as the first endpoint of the deletion for several reasons, first, the *Cftr* gene lies distal to the LOH region in human 7q31.1-7q31.2 and is syntenic on mouse chromosome 6⁵² (See Figure 1), second, the mouse homologue of *Cftr* is available in our laboratory and DNA probes have been developed⁵³. These reagents have facilitated the construction of the *HPRT* cassettes and in the identification of homologous integration events following electroporation of the cassettes into ES cells. *HPRT* cassettes lacking the 3' *HPRT* sequences have been cloned into the *Cftr* gene (See Figure 3.) in both orientations. These constructs have been designed as conventional replacement vectors with positive (neo)

selection. These vectors have been electroporated into AB2.1 ES cells. Targeted clones have been identified by Southern analysis of isolated clones grown in 96 well plates according to standard protocols used in the laboratory.

Figure 3



Targeting the second deletion end points. I have chosen the anonymous DNA markers *D6MIT138* and *D6MIT170* as the proximal and distal end points for the deletions as there were no candidate mouse genes from this region and the resulting deletion locus extends beyond the region syntenic to the human LOH region. I have obtained mouse λ clones containing the *D6MIT138* and *D6MIT170* markers from a mouse genomic library. A detailed restriction map of the *D6MIT138* and *D6MIT170* loci have been constructed from these phage. Two

conventional insertion vectors have been constructed for each locus utilizing the *HPRT* cassette lacking the 5' *HPRT* exons and containing the puromycin selectable marker such that both possible orientations are represented (See Figures 4 and 5).

Figure 4

D6MIT138-KOs

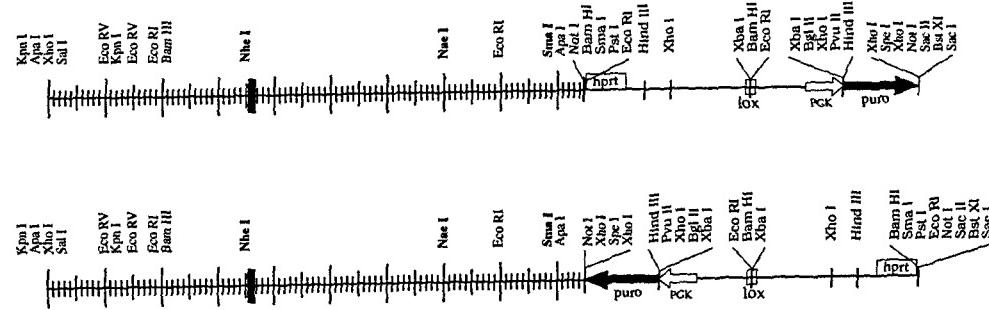
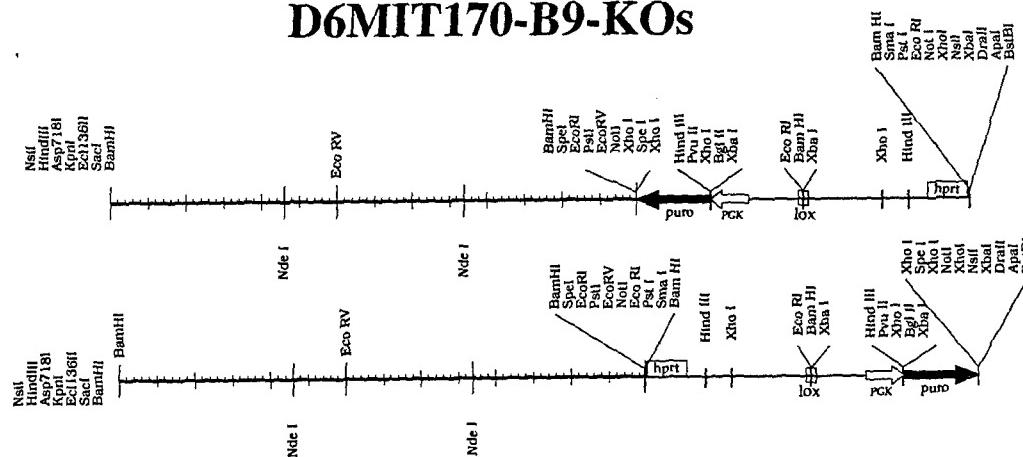


Figure 5

D6MIT170-B9-KOs

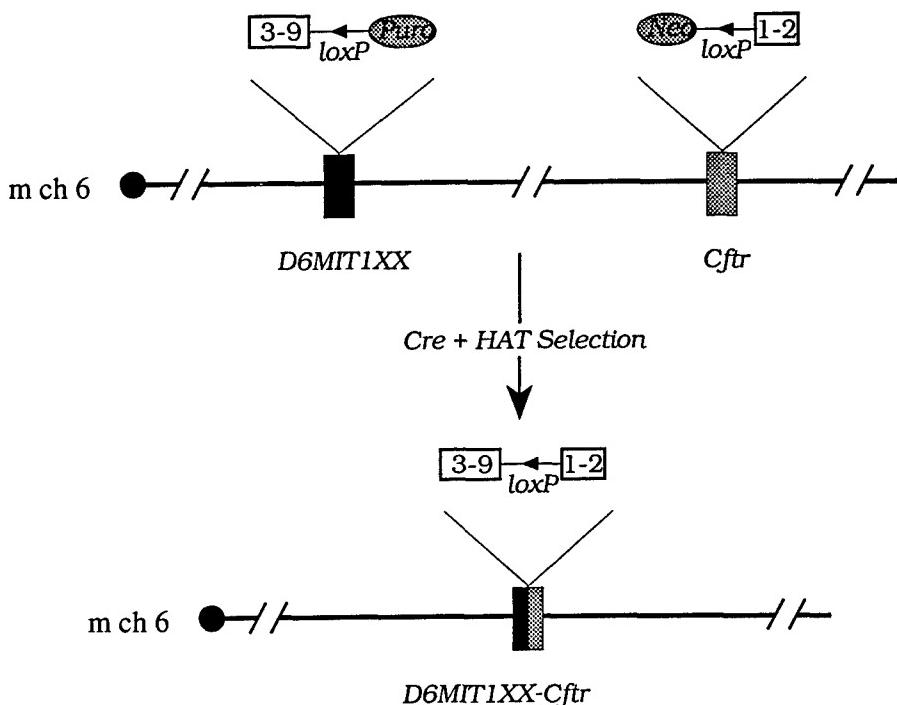


These vectors have been transfected into AB2.1 ES cells containing each of the targeted *Cftr* loci obtained from the above experiment. All four possible combinations of cassette orientations have been utilized. Furthermore, multiple independent doubly targeted clones, representing each of the truncated *HPRT* minigene orientations, have been collected to insure both *cis* and *trans* representation.

Obtaining clones with deletions. Construction of a deletion between *D6MIT138* or *D6MIT170* and *Cftr* in the absence of prior knowledge of the gene orientations will require that all four possible arrangements of the truncated *HPRT* minigenes must be tested, only one of which will generate deletions⁵⁰ (See Figure 6). Double targeted ES cells have been expanded and electroporated with a Cre expression vector. Cre recombinase directed functional *HPRT* recombinants were selected for under HAT selection. Our laboratory has demonstrated that the frequency for Cre mediated recombination between distant *loxP* sites on the same chromosome to vary from 10^{-4} to 10^{-5} per cell electroporated with the cre expression cassette. There does not appear to be a direct relationship between the observed recombination frequency and distance between *loxP* sites as a 1Mb deletion was obtained at a greater frequency than a 90kb deletion. These experiments indicate that construction of a 1cM deletion between *Cftr* and *D6MIT138* or *D6MIT170* is plausible utilizing the described methodology.

Identification of deletions, versus other recombination consequences of incorrect *HPRT* minigene orientation combinations, is obtained through testing the HAT resistant colonies for sensitivity to G418 and puromycin. HAT resistant colonies from incorrect *HPRT* minigene orientations will remain resistant to one or both antibiotics.

Figure 6



However, HAT resistant colonies from correct *HPRT* minigene orientations in the *cis* configuration will have deleted both antibiotic resistance genes and will therefore be sensitive to both drugs (See Figure 6). DNA from HAT resistant, puromycin and neomycin sensitive colonies have been analyzed by Southern analysis to confirm the authenticity of the deletion. ES cells containing the deletion have been micro-injected into day 3.5 blastulas and placed into pseudo-pregnant female surrogates⁵⁴⁻⁵⁶. Chimeric mice are currently being tested for germ line transmission of the deletion which will be confirmed by Southern analysis of DNA isolated from pups begotten from the chimeric parent. The

tumor suppressor gene will be identified through a retroviral mediated *in vitro* assay in tissue culture using cells derived from these mice.

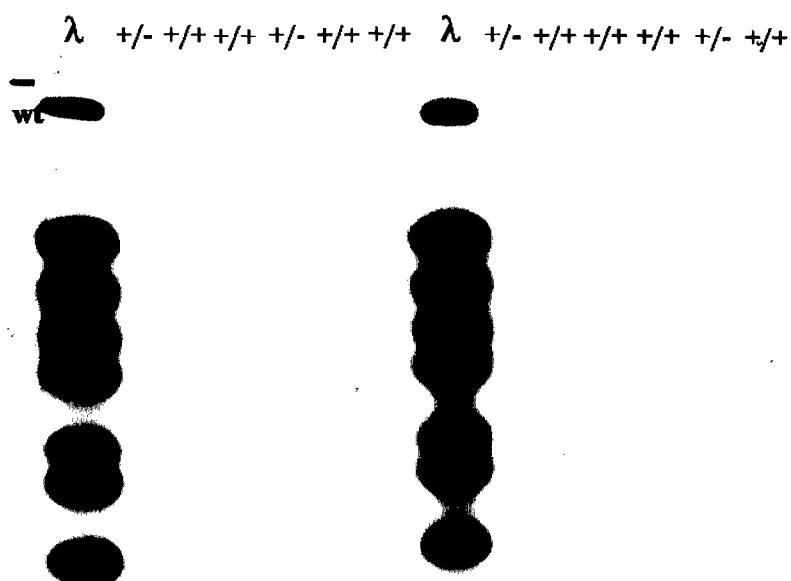
Results and Discussion

Construction of mice with tumor suppressor gene deletions.

Large deletion of chromosome 6.

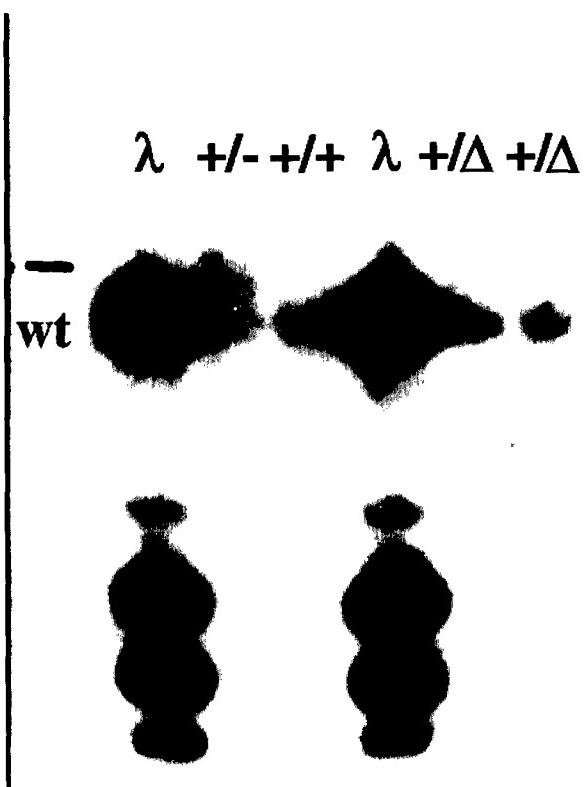
Vectors for the construction of the deletion were constructed (See Figures 3-5). The Cftr constructs were electroporated into ES cells, cultured under neomycin selection, neomycin resistant colonies picked and analyzed by Southern analysis for homologous integration of the Cftr construct. A number of homologous recombinant cell lines were identified by Southern analysis (See Figure 7).

Figure 7



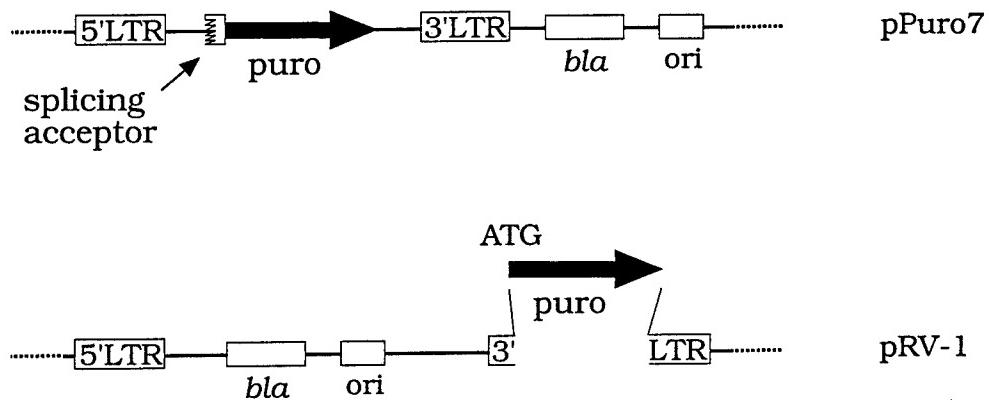
Cell lines from each Cftr deletion orientation were electroporated with one of the D6MIT138 or D6MIT170 constructs such that each of the possible combination of orientations were represented. These cells were cultured under puromycin selection, pooled and electroporated with the plasmid pOG231 that directs the expression of Cre recombinase. These cells were then cultured under HAT selection and colonies were picked. These cell lines were tested for sensitivity to puromycin and neomycin. DNA from HAT^r, puro^s and neo^s cell lines was subjected to Southern analysis to confirm the deletion (See Figure 8). Cell lines confirmed to carry the deletion in chromosome 6 have been micro-injected into day 3.5 blastocysts and chimeric mice have been generated.

Figure 8



Two retroviruses will be used with primary fibroblasts and mammary epithelial cells to inactivate and identify the tumor suppressor gene. The first virus, pPuro7, was obtained from Lexicon Genetics (Houston, Texas) and is shown in Figure 9. When the pPuro7 retrovirus integrates within an intron of an expressed gene a fusion transcript is generated and puromycin resistance may be obtained. We expect many such integrations to inactivate the gene within which the retrovirus has integrated. The second virus, pRV-1, has been constructed (See Figure 9). pRV-1 has been designed such that upon viral integration into an exon of an expressed gene puromycin resistance may be obtained. We expect a higher frequency of puromycin resistant integration events to be located at 5' exons. Furthermore, integrations into 5' exons are very likely to lead to the inactivation of the gene into which the virus has integrated.

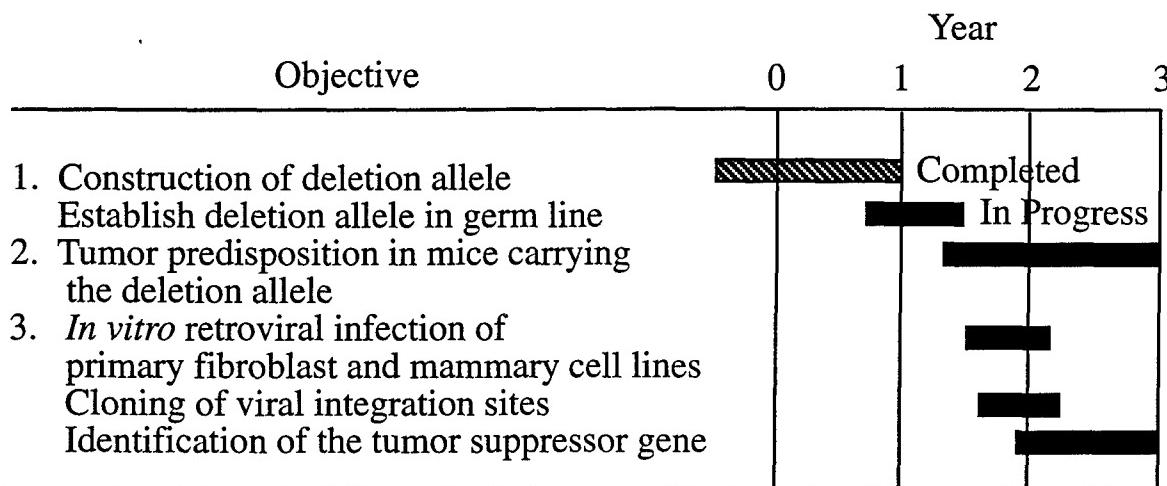
Figure 9



Recommendations in relation to the Statement of Work

The research proposed in the Statement of Work outlined in the proposal has progressed largely as originally stated. Objective 1, the construction of the deletion allele has been completed as proposed. The deletion alleles have been and are being micro-injected into blastocysts for the production of chimeric mice. Chimeric mice have been obtained for one of the deletions and these mice are being mated to test for germ line transmission of the deletion. Tumor predisposition studies of mice carrying a deletion over the interval within chromosome 6 will begin immediately following successful germ line transmission.

The third objective, MMTV tumor induction and molecular analysis of MMTV induced tumors to identify the tumor suppressor gene has been modified. This objective will now be met by an *in vitro* retroviral assay, described above. The *in vitro* assay for the identification of the tumor suppressor gene is expected to proceed more rapidly than the previously proposed method. Furthermore, the *in vitro* assay has the additional advantage of minimizing the number of animals needed for the completion of this proposal. A modified time line for this project is given below.



Conclusions

The research completed thus far, the vector constructions and homologous integrations, the engineering of the deletions within chromosome 6, the injection of these cells into blastocysts and the production of chimeric mice are crucial steps in the identification of the tumor suppressor gene that lies within the deletion interval. These initial steps will allow for the production of mice heterozygous for the deletion intervals. These mice will then be mated to mice containing the neomycin resistance gene at the *Cftr* locus and primary cell lines will be derived from embryos from these matings. These primary cell lines are crucial for the *in vitro* retroviral assay proposed for the identification of the tumor suppressor gene. The tumor suppressor identified through the *in vitro* retroviral assay will be sequenced and characterized. These characterizations may reveal fundamental insights into the mechanism of a large fraction of sporadic breast cancers. Ultimately these studies may lead to important diagnostic assays and novel chemotherapeutic or gene replacement treatments.

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1994-present

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Prizes and Awards

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Prizes and Awards Continued

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Professional Memberships

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The American Society for Microbiology

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Publications

Dietrich, K., Kelly A. Skinner, **Henry B. Skinner**, Dale A. Parks and Andrew J. Ghio (1997) Xanthine Oxidase Functions as a Ferric Reductase. In Preparation - to be submitted to the Journal of Clinical Investigation.

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Publications Continued

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Patent Applied For

Monoclonal and Polyclonal Antibodies Against Recombinant Human Xanthine Oxidase, Method for their use and a Kit Containing the same. Applicants: Dale A. Parks, Kelly A. Skinner and **Henry B. Skinner**. Application Serial No. 08/455,148